Protease-Activated Receptor-2 (PAR-2) Expression in Human Fibroblasts is Regulated by Growth Factors and Extracellular Matrix

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Many cell types express a membrane receptor, activated by trypsin-like proteases, termed protease-activated receptor-2 (PAR-2). Previous studies describing PAR-2 expression on fibroblasts have been conflicting. In this report, we investigated *in vitro* PAR-2 expression on several fibroblast cell lines using flow cytometry, immunohistology, and immunoblots of cell lysates. Consistent PAR-2 expression was detected in cultured fibroblasts, although we observed heterogeneity of cellular expression among the cell lines. Some fibroblast lines expressed PAR-2 predominantly as an intracellular protein with differing cytoplasmic staining patterns, whereas other fibroblast lines displayed PAR-2 primarily as a cell surface receptor. Immunoblots of cell lysates with polyclonal anti-PAR-2 demonstrated a 44 kDa band, the predicted molecular weight for the PAR-2 core protein. Furthermore, we noted that expression of PAR-2 was subject to regulation. Fibroblasts grown within a collagen matrix downregulated receptor expression whereas increased PAR-2 expression was observed by the addition of fibroblast growth factors PDGF-BB and TGF-β. This study may explain the previous inconsistencies in PAR-2 expression observed on tissue fibroblasts. Results indicate that the degree of fibroblast proliferation, attenuated by extracellular matrix and upregulated by growth factors, influences whether fibroblasts express PAR-2 and, thus, would be responsive to protease signaling.

Key words: protease activated receptors/fibroblasts/fibrosis/PDGF/collagen
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It is well established that serine proteases, such as thrombin, can exert cytokine-like effects on human tissue fibroblasts. This has largely been attributed to their ability to initiate signaling via protease-activated receptors (PAR) expressed by tissue fibroblasts (Hollenberg and Compton, 2002). More recently, evidence has emerged demonstrating the capacity of mast cell-derived proteases such as tryptase to activate fibroblasts (Ruoss *et al*, 1991). Initially, the mechanism by which tryptase stimulated fibroblasts was unknown, especially since tryptase lacked the ability to activate the classical thrombin receptor (PAR-1). Shortly thereafter, following the discovery of a second protease-activated receptor (PAR-2), several independent investigators demonstrated that tryptase can cleave and activate PAR-2 (Corvera *et al*, 1997; Mirza *et al*, 1997; Molino *et al*, 1997; Schechter *et al*, 1998; Steinhoff *et al*, 1999; Compton *et al*, 2001; Akers *et al*, 2002). PAR-2, however, was noted to be expressed predominantly on cells of epithelial or endothelial origin, with prior reports documenting a virtual absence of PAR-2 mRNA and protein expression on fibroblasts (Santulli *et al*, 1995; D'Andrea *et al*, 1998). Further studies by other investigators demonstrated weak expression on dermal fibroblasts but in contradistinction more consistent PAR-2 expression in lung parenchymal or airway-derived fibroblasts (Schechter *et al*, 1998; Steinhoff *et al*, 1999; Akers *et al*, 2002). Thus, the mechanism by which mast cell-derived tryptase activates fibroblasts has remained puzzling and led some investigators to postulate that a separate (yet to be identified) tryptase-activated receptor exists on fibroblasts (Akers *et al*, 2002).

The uncertainty concerning fibroblast expression of PAR-2 remains largely unresolved despite the considerable body of evidence that has accrued portraying a role for mast cell–fibroblast interactions, and specifically, tryptase activation of fibroblasts (Gruber *et al*, 1997). Some insight into this ambiguity surfaced with the finding of PAR-2 expression *in situ* specifically on proliferating fibroblasts within the stroma immediately surrounding neoplasms or healing wounds (D'Andrea *et al*, 2001). These observations raise the intriguing possibility that human fibroblasts have the capacity to express PAR-2 as an inducible membrane protein, perhaps stimulated by growth factors and regulated by extracellular matrix. In this investigation, we demonstrate the capacity of human fibroblasts to express PAR-2 *in vitro* and characterize the factors that govern this expression.

Abbreviation: PAR, protease-activated receptors

Results

PAR-2 expression on cultured fibroblasts Immunohistological analysis revealed consistent PAR-2 staining, although distinct patterns were observed. Two cell lines of fibroblasts were selected for more detailed analysis as differences in PAR-2 expression emerged. Fibroblasts 4392A expressed a substantial amount of membrane staining as compared to 2261B that predominantly expressed PAR-2 as an intracellular protein (Fig 1*A*). Furthermore, different patterns of intracellular staining were evident. Within the 2261B fibroblasts, a proximal perinuclear pocket staining pattern was observed (Fig 1*B*, *left panel*). Other cells displayed a fine and coarse granular cytoplasmic pattern (Fig 1*B*, *center panel*) with vesicular intracytoplasmic staining (Fig 1*B*, *right panel*). The staining of vesicle-like structures with PAR-2 was similar to that previously described to be associated with the golgi apparatus in rat kidney cells (Dery *et al*, 1999).

Flow cytometry analysis The fibroblasts 4392A and 2261B were subjected to flow cytometry following dispersion from the culture flasks for quantitative evaluation of PAR-2 expression. Flow cytometry analysis confirmed differences in the distribution of PAR-2 between 4392A and 2261B cells (Fig 2). Whereas the majority (70%) of 4392A cells expressed surface PAR-2, only a subset (25%) of 2261B cells were positive ($p = 0.0001$, $N = 7–8$). Moreover, the surface level of PAR-2 was significantly increased on a per cell basis, as indicated by the intensity of fluorescence (noted by both microscopic and flow cytometric analysis). Although 2261B did not demonstrate prominent surface staining, intracellular expression was strong in these cells (Fig 2*A*). Interestingly, despite the markedly different anatomical localization of PAR-2, the total level (surface plus intracellular) of PAR-2 was not significantly different between 4392A and 2261B cells (85% *vs* 79%, respectively, $p = 0.3$, $N = 8–10$). The total level of fluorescence on a per cell basis was also not altered.

Two-color analysis by flow cytometry revealed that most of the cultured 4392A and 2261B cells expressed smooth muscle actin in conjunction with PAR-2 (Fig 3), possibly reflecting a myofibroblast-like phenotype. In addition, a correlation between PAR-2 expression and proliferation was suggested by a double staining pattern observed on fibroblasts co-expressing PAR-2 and Ki67, a nuclear cell proliferation-associated marker (data not shown).

Immunoblot analysis of PAR-2 expression Immunoblot analysis of PAR-2 protein expression on detergent-soluble lysates derived from various fibroblast cell lines originating from different organs is demonstrated in Fig 4. The survey included different fibroblast cultures from adult skin (including 2261B), neonatal skin, fetal skin (including 4392A), adult lung, and fetal lung. The fibroblast cultures all expressed measurable levels of PAR-2. In this analysis, we observed PAR-2 core protein at the expected molecular size of 44 kDa (Fig 4), as well as higher molecular weight immunoreactive bands (not shown). The higher molecular weight forms may possibly represent fully glycosylated receptors

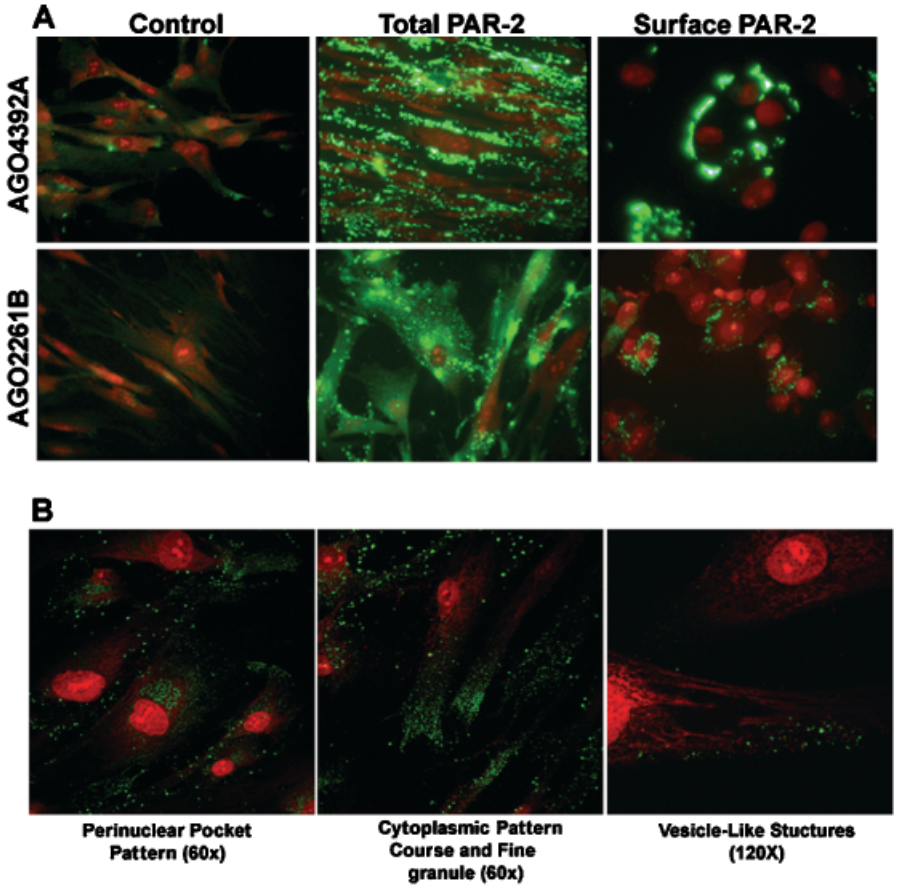

Figure 1
Cellular distribution of PAR-2 in fibroblast lines. (*A*) Fibroblasts 4392A (*upper panels*) and 2261B (*lower panels*) were grown on slides, fixed, permeablized and stained with either isotype-matched controls (*left panels*) or mAb to PAR-2 *in situ* (*center panels*). In addition, fibroblasts were stained in suspension to monitor surface PAR-2 expression, deposited onto slides, and counterstained with propidium iodide (PI) (*right panels*). Fluorescence microscopy revealed a pattern of PAR-2 expression primarily on the surface in 4392A fibroblasts, whereas predominant intracellular staining was observed in 2261B fibroblasts. Original magnification, $\times 40$ (*B*) Sub-confluent cultures of 2261B fibroblasts grown in chamber slides were stained with mAb to PAR-2 and counterstained with PI. Analysis by confocal microscopy revealed distinct patterns of intracellular PAR-2 including pockets distributed proximal to the nucleus (left panel) as well as cytoplasmic fine and course granules (*central panel*). Higher magnification of the coarse granules showed them to resemble vesicles (*right panel*).

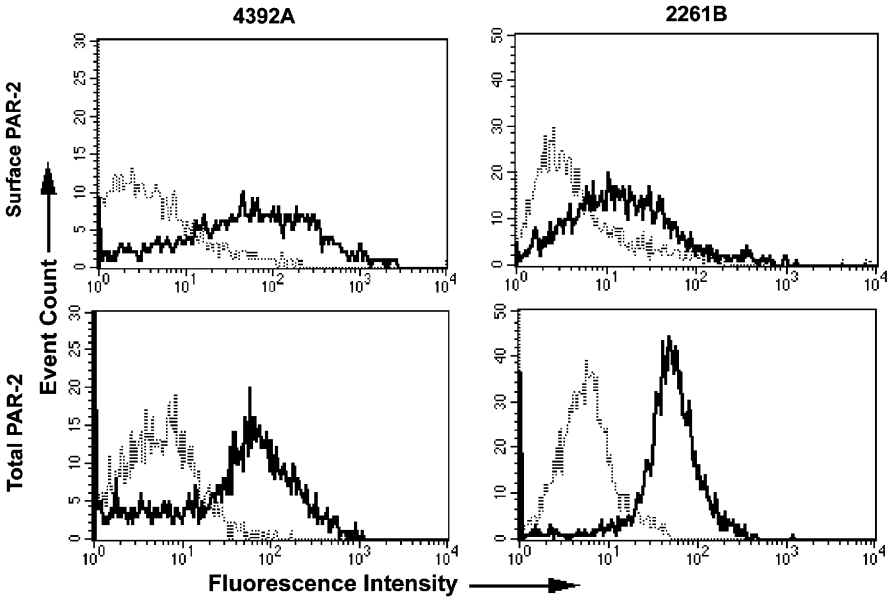

Figure 2
Flow cytometric analysis of differential PAR-2 expression by fibroblast lines. Fibroblasts 4392A (*left panels*) and 2261B (*right panels*) were stained in suspension for surface (*upper panels*) PAR-2 expression and total cellular PAR-2 expression using permeablized cells (*lower panels*), as described in methods section. ($n \geqslant 7$ for each group).

as previously described (Compton *et al*, 2001). Pre-absorption experiments using anti-PAR-2 blocking peptide, however, failed to diminish the expression of these higher immunoreactive bands. Thus, we have focused on the 44 kDa form since pre-treatment of anti-PAR-2 with its specific blocking peptide completely abrogated this immunoreactive band (data not shown). Variability of PAR-2 expression in quantitative terms seemed largely independent of tissue source and chronological age of tissue donor (data not shown). The number of population doublings also did not appear to affect PAR-2 expression *in vitro*, with stable patterns noted up to 37 PDLs (data not shown).

PAR-2 expression is inducible and regulated Because PAR-2 expression was observed as a consistent property of all fibroblasts studied under cultured conditions, but yet rarely expressed normally *in situ*, we next investigated the possibility that extracellular matrix regulated PAR-2 expression. Our initial efforts involved culturing the cells layered onto collagen to allow a 2-dimensional exposure to extracellular matrix. No alteration in PAR-2 expression was observed (Fig 5*A*). We also studied the effect of cultures exposed in a similar manner to fibronectin and likewise observed minimal effects (Fig 5*A*). In an effort to more closely replicate fibroblasts *in vivo*, we next embedded the fibroblasts within a three-dimensional collagen matrix. Using this culture system, we noted a marked decrease in PAR-2 expression within 24 h of exposure that continued to diminish over 48 h (Fig 5*B*). PAR-2 expression was essentially abrogated by 48 h in both the 4392A and 2261B cell lines,

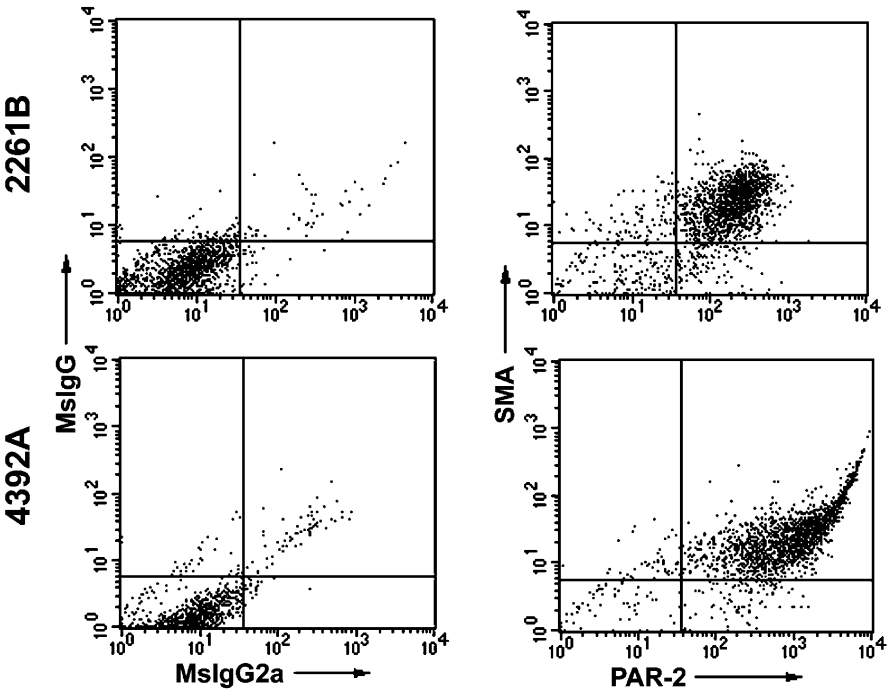

Figure 3
Fibroblast lines co-express PAR-2 and smooth muscle actin (SMA) indicative of myofibroblast morphology. Fixed and permeablized 2261B fibroblasts (upper panels) and 4392A fibroblasts (*lower panels*) were double stained with isotype controls (*left panels*) or antibody to PAR-2 and SMA (*right panels*) and analyzed by flow cytometry as described in Materials and Methods. Figure shown is representative of two experiments giving identical results.

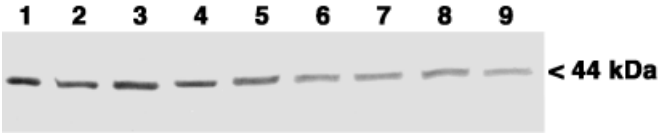

Figure 4
Immunoblots demonstrate PAR-2 expression in a wide survey of different fibroblast cell lines. PAR- 2 was visualized using goat polyclonal antibody recognizing NH-terminus of human PAR-2 in cell lysates (prepared by NP-40 detergent lysis buffer of confluent cells) and loaded with protein content 3–10 μg per lane. The fibroblasts originated from varying organs and donors, including fetal male lung MRC-5 (*lane 1*), fetal female lung AGO4393 (*lane 2*), fetal female skin AGO4392 (*lane 3*), fetal male lung AGO4526 (*lane 4*), fetal male skin AGO4525 (*lane 5*), adult 35 female lung AGO2603 (*lane 6*), adult 35 female skin AGO2602A (*lane 7*), adult 61 male lung AGO2262 (*lane 8*), adult 61 male skin AGO2261A (*lane 9*). Note that the core protein at 44 kDa is present in all the fibroblasts.

although a slight variation in kinetics was noted between the two lines (Fig 5*B*). Prolonged culture over 10 days had minimal effect, with no further appearance or re-emergence of PAR-2 after 72 h (data not shown). In further support of the data obtained by immunoblot analysis, immunocytochemistry confirmed downregulation of PAR-2 after fibroblasts were embedded in a collagen matrix system (Fig 5*C*). In this analysis, decreases in both intracellular and surface PAR-2 expression were clearly evident on 2261B and 4392A cells, respectively. In order to mimic *in vivo* conditions, we further studied PAR-2 expression in an organotypic keratinocyte:fibroblast 3-dimensional matrix culture system as previously described (Garlick and Taichman, 1994; Gruber *et al*, 1997). Immunostaining of frozen sections demonstrated keratinocyte but no fibroblast staining (data not shown).

Since collagen three-dimensional culture systems appeared to downregulate the PAR-2 expression mimicking the typical *in vivo* situation, we next studied the effect of growth factors on upregulating PAR-2 expression. Our initial efforts monitored the effects of platelet-derived growth factor-BB and transforming growth factor-β in stimulating PAR-2 expression on fibroblasts cultured under standard conditions. As indicated in Fig 6, PAR-2 expression was again noted under basal conditions but increased after a 72 h exposure to TGF-β and substantially more when exposed to PDGF-BB. Treatment with both growth factors together yielded a small additional effect (Fig 6). In contrast, exposure to tumor necrosis factor-α and interleukin 1-α resulted in no alteration in fibroblast PAR-2 expression (data not shown). A dose response to PDGF-BB (Fig 7), as well as a time course (not shown) for upregulation of PAR-2 expression was observed in cultured fibroblasts. A dose dependency was observed to PDGF-BB for concentrations up to 30 ng per mL (Fig 7), with a time course indicating maximal expression by 24 h (not shown). We next investigated the effect of these growth factors when added to fibroblasts in three-dimensional collagen matrix where PAR-2 expression was downregulated. As shown in Fig 8, PDGF-BB was capable of stimulating PAR-2 fibroblast expression within the collagen matrix culture system. In fact, we noted that by 48 h exposure to PDGF-BB the expression of PAR-2 was similar to that noted when the cells were grown on plastic in the absence of collagen (Fig 8). Finally, the effect of a three-dimensional collagen matrix and PDGF-BB on fibroblast

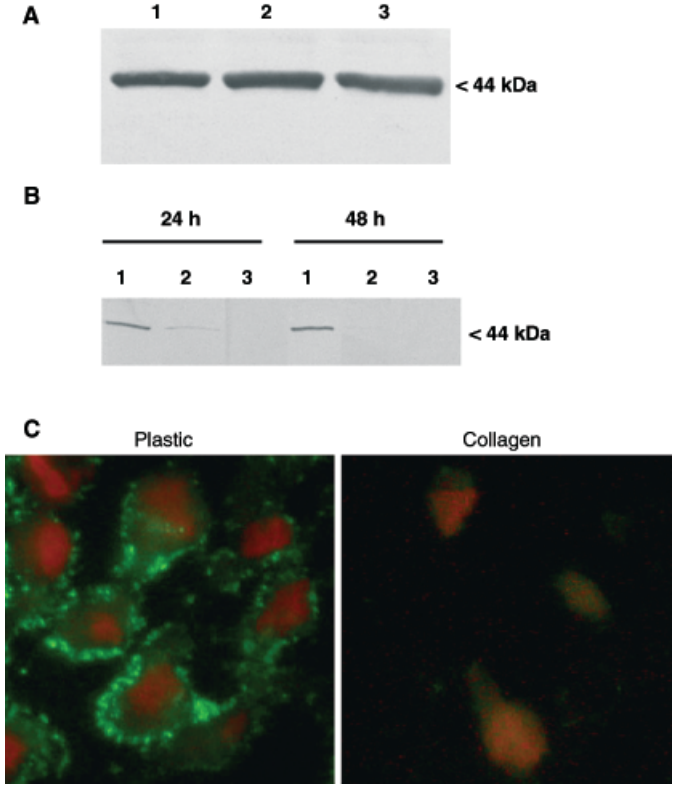

Figure 5
Effect of collagen on PAR-2 expression. (*A*) Immunoblot demonstrating PAR-2 expression of fibroblasts cultured as monolayers on the surface of extracellular matrix. Fibroblasts 2261B cultured using standard conditions in plastic dishes (*lane 1*) were compared to fibroblasts layered on type I collagen (*lane 2*) or fibronectin (*lane 3*) for up to 72 h. Note the lack of any change in PAR-2 expression despite varying culture conditions. The immunoblot shown is representative from three separate experiments. (*B*) Immunoblot of PAR-2 expression in fibroblasts embedded in collagen matrices. Cells cultured *within* three-dimensional collagen gels exhibited marked attenuation of 44 kDa PAR-2 core peptide expression as compared to standard culture conditions. Shown is fibroblast PAR-2 expression on plastic dishes in serum-free media as per standard culture conditions (*lanes 1*, at time ponts indicated), compared to PAR-2 expression after initiating growth within collagen gels at 24 and 48 h in the presence and absence of serum (*lanes 2* and *3*; 24 and 48 h as shown). The immunoblot is representative from four separate experiments. (*C*). Immunostaining of 2261B fibroblasts indicating PAR-2 expression under standard culture conditions (*left panel*) compared to growth within collagen matrices (*right panel*). Fibroblast cell lines were grown in collagen for 48 h, harvested as described in Materials and Methods, and then deposited onto slides, fixed, permeablized and stained with polyclonal antibody to PAR-2 followed by anti-goat IgG-Alexa 488. Note the intense PAR-2 staining when cultured under standard monolayer conditions in plastic dishes and the marked attenuation of staining when cultured within collagen matrices.

PAR-2 mRNA was analyzed by RT-PCR (Fig 9). There is a clear PDGF-induced increase in PAR-2 mRNA both in cells grown on plastic or imbedded in collagen. Although growth in a 3-D collagen matrix largely eliminated expression of the PAR-2 protein, however, the mRNA remained at a basal level (Fig 9).

Discussion

A myriad of reports over several decades have described mast cell hyperplasia arising in fibrosing tissue regardless of the underlying diagnosis (Gruber, 1995; Gruber *et al*, 1997). Both in normal reparative and pathologic wound healing,

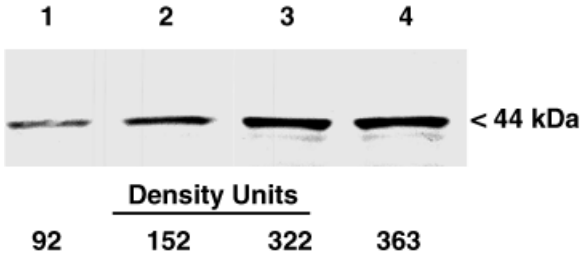

Figure 6
Immunoblots of fibroblasts indicating the effect of growth factors on PAR-2 expression. Cultures of adult dermal fibroblasts were grown for 9 days without feeding, washed, and treated for 72 h with serum-free medium containing no growth factors (*lane 1*), TGF-β at 5 ng per mL (*lane 2*), PDGF-BB at 30 ng per mL (*lane 3*), or both PDGF-BB and TGF-β (*lane 4*). Note increased 44 kDa PAR-2 expression with the addition of the growth factors. The immunoblot shown is representative from five separate experiments.

mast cells have been found in increased numbers at sites of excessive tissue remodeling (Lee Choi and Claman, 1987; Rothe and Kerdel, 1991). This observation lends support to the notion that mast cells and fibroblasts interact in a manner leading to a fibrogenic cascade. Numerous cell coculture studies have provided evidence to substantiate this concept. Taken together, the published studies document that mast cell products have the capacity to augment fibroblast proliferation, chemotaxis, matrix synthesis, and myofibroblast transformation (Gruber, 2003).

A major product produced uniquely by human mast cells is the serine protease tryptase (20). Tryptase is capable of activating fibroblasts and thus has been considered a potent fibrogenic factor (Gruber *et al*, 1997). Furthermore, the mechanism by which tryptase activates cells has been attributed to its capacity to cleave PAR-2 (Corvera *et al*, 1997; Mirza *et al*, 1997; Molino *et al*, 1997; Schechter *et al*, 1998; Steinhoff *et al*, 1999; Compton *et al*, 2001; Akers *et al*, 2002), a seven transmembrane G-protein receptor distinguished by a unique mechanism of self-activation following enzymatic cleavage (Macfarlane *et al*, 2001).

Although substantial data demonstrate the ability of tryptase to activate fibroblasts via a proteolytic mechanism, previous studies depict minimal if any expression of its putative receptor PAR-2 on tissue fibroblasts under normal conditions *in vivo* (Santulli *et al*, 1995; D'Andrea *et al*, 1998). However, in pathologic states or during wound healing,

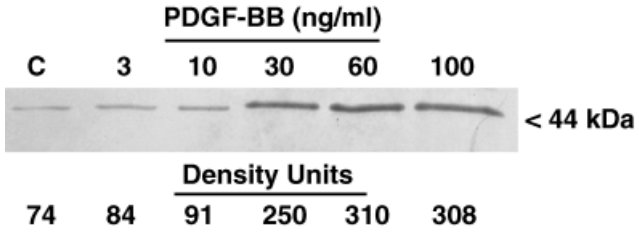

Figure 7
Immunoblot demonstrating inducible PAR-2 expression in a dose-dependent fashion in response to exogenous PDGF-BB. Cultures of 2261B fibroblasts were grown on tissue culture plates in serum-free media with varying amounts of PDGF-BB (3–100 ng per mL) for 72 h; cells were then lysed for immunoblotting to assess a dose response for PAR-2 expression. Densitometric analysis of the 44 kDa core receptor is shown as absorbance units. The immunoblot shown is representative from three separate experiments.

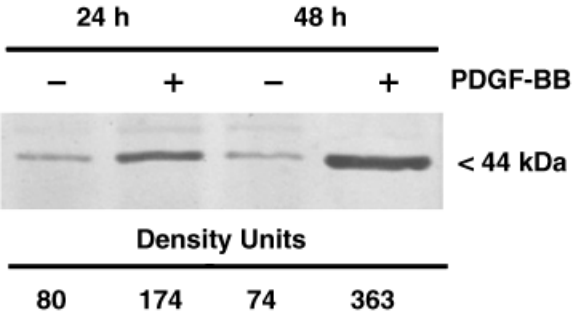

Figure 8
Immunoblot demonstrating inducible PAR-2 expression with PDGF-BB despite embedding fibroblasts within a collagen matrix. Fibroblast cultures 4392A were grown as described in Materials and Methods in three-dimensional collagen gels to minimize PAR-2 expression for varying incubation periods as indicated. PDGF-BB at 30 ng per mL was added at the time of gel formation (n = 3). Densitometric analysis of the 44 kDa core receptor is shown as absorbance units in this representative immunoblot at 24 and 48 h as indicated.

D'Andrea *et al* (2001) noted distinct PAR-2 expression on stromal fibroblasts immediately surrounding the underlying process. Thus, it seems plausible that although quiescent tissue fibroblasts constitutively express minimal PAR-2 *in vivo*, conditions that promote fibroblast proliferation or activation might induce PAR-2 expression. In this report, we present experimental data to confirm this hypothesis. We specifically identified PDGF-BB and TGF-β as potent inducers of fibroblast PAR-2 expression and provide evidence to substantiate that extracellular matrix may downregulate its expression.

Previous publications have differed in terms of fibroblast capacity to express PAR-2. Initial studies characterizing the distribution of PAR-2 noted relatively high levels in certain lung fibroblasts but absent or much lower levels in dermal fibroblasts (Schechter *et al*, 1998; Akers *et al*, 2002). Certainly, methodological differences, as well as possible fibroblast heterogeneity, and staining techniques may have accounted for some of these different observations. Our contention that PAR-2 expression is subject to regulation was initially supported in endothelial cells by the finding of

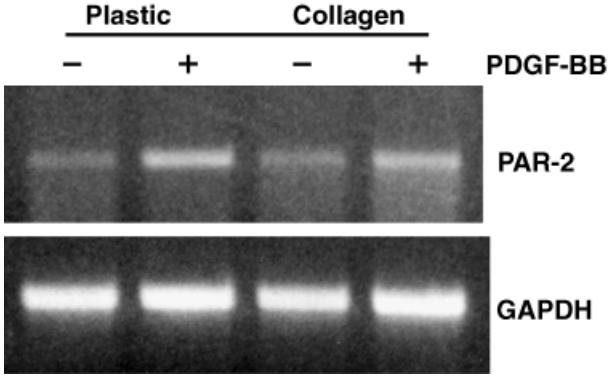

Figure 9
RT-PCR demonstrating increased PAR-2 mRNA expression with PDGF-BB in fibroblasts on plastic and within a collagen matrix. Fibroblasts (4392A) were grown on plastic as described in Materials and Methods or in three-dimensional collagen gels to minimize PAR-2 expression for 24 h. PDGF-BB at 30 ng per mL was added at the time of collagen gel formation.

inducible PAR-2 expression following exposure to inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1 (Nystedt *et al*, 1996). Thus, the observed differences in PAR-2 expression between lung and skin fibroblasts may primarily reflect differences in the growth conditions provided by their native tissues *in vivo*, as well as differences in their responses to culture conditions *in vitro*.

Our data studying human fibroblasts *in vitro* demonstrates unequivocally PAR-2 expression in numerous cultures of both lung and skin fibroblasts (Fig 4). This included different fibroblast cultures from adult, neonatal, and fetal skin, as well as adult and fetal lung. PAR-2 expression seemed largely independent of tissue source, age of donor, and number of population doublings in culture. We also observed that MRC-5 fetal lung fibroblasts, which have previously been reported to respond to tryptase as a mitogen (Cairns and Walls, 1997), strongly expressed PAR-2. Similarly, the foreskin fibroblasts AG01523, which we previously demonstrated could be activated by tryptase (Gailit *et al*, 2001), also expresses PAR-2 and is supportive evidence that the expressed PAR-2 is functional, although additional studies will be required to confirm this notion.

Since the expression of PAR-2 appeared to be a consistent property of cultured fibroblasts, we investigated the regulation of PAR-2 expression to further understand the relative absence of PAR-2 under normal condition, *in vivo*. Many phenotypic properties change during the differentiation of fibroblasts into myofibroblasts, and the expression of PAR-2 may be among them (Dery *et al*, 1998; D'Andrea *et al*, 2001; Akers *et al*, 2002). We presume that myofibroblast transformation is associated with PAR-2 expression, since TGF-β1 and PDGF-BB, two growth factors most closely linked to induction of myofibroblast proliferation and differentiation *in vivo*, stimulated PAR-2 expression (Desmouliere *et al*, 1993; Grinell, 2003). In addition, we documented a marked reduction in PAR-2 expression in fibroblasts when embedded within an extracellular matrix, thus simulating normal organ tissue conditions with fibroblasts sustained in a low proliferative state (Grinell, 2003).

In summary, this study provides definitive data supporting the concept that fibroblasts are capable of expressing cell surface PAR-2 and thus becoming responsive to tryptase activation by mast cells and this expression is subject to downregulation by local microenvironmental conditions and induction by paracrine growth factors. The flow cytometry, as well as the immunohistological studies, indicate that the PAR-2 receptor peptide is present in both the cytoplasm and expressed on the membrane of these fibroblasts. Additional studies will thus be required to precisely determine and characterize the functionality of this receptor under varying conditions. Future studies concerning the regulation of PAR-2 expression and its function in disorders characterized by abnormalities in fibroblast–mast cell interactions should yield further insight into the specific role of PAR-2 in the disease process.

Materials and Methods

Cell culture The following cultures of normal human fibroblasts were obtained from the National Institute on Aging, Aging Cell Culture Repository, Coriell Institute for Medical Research (Camden, NJ): adult lung (AG0-2262, 2603), fetal lung (AG0-4393, 4526), adult skin (AG0-4145, 5186, 4453, 10047, 2261B, 6290, 7471, 2602, and 5838), neonatal skin (AG0-1523, 8498), and fetal skin (AG0-4392A, 4525). Each culture of lung fibroblasts (AG0-2262, 2603, 4393, 4526) was matched with a corresponding culture of skin fibroblasts (AG0-2261B, 2602, 4392A, 4526) obtained from the same donor. Three other cultures of fetal lung fibroblasts, MRC-5, IMR-90, and WI-38, were also obtained from the Coriell Institute. All fibroblast cultures were grown in minimum essential medium containing Earle's salts, and 26 mM sodium bicarbonate, 25 mM HEPES, pH 7.3 (Life Technologies, Gaithersburg, Maryland), supplemented with non-essential amino acids (Life Technologies), 10% fetal bovine serum (HyClone Laboratories, Logan, Utah), and 100 U per mL penicillin and 100 μg per mL streptomycin. The serum-free medium (SF-MEM) used in the experiments described below was identical to the fibroblast growth medium except that it contained 2 mg per mL BSA (low endotoxin, fatty acid free; ICN Biomedical Research Products, Costa Mesa, California) instead of serum. The population doubling (PDL) of the fibroblast cultures used ranged from 15 to 25, but several cultures with high numbers of population doublings were also included for specific experiments. All cell cultures were maintained in an atmosphere of air and 5% CO_2 at 37°C.

Immunofluorescence Studies For PAR-2 surface analysis by flow cytometry, cells were detached using non-enzymatic techniques (Cell Stripper, Cellgro, Fisher Scientific, Springfield, New Jersey), washed in staining buffer (PBS (phosphate-buffered saline), 0.1% sodium azide, 1% BSA), and then incubated with either a monoclonal (clone sc-13504 at 1 μg per $\leqslant 10^6$ cells) or polyclonal PAR-2 Ab (clone sc-82061 at 0.3 μg per $\leqslant 10^6$ cells) or with isotype-matched control Abs (Becton-Dickinson/Pharmingen, Sunnyvale, Califonia; Sigma, St Louis, Missouri) for 45 min on ice. Both anti-PAR Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, California), were generated against N-terminal peptides, and both reacted with cleaved and uncleaved PAR-2. After incubation with primary Ab, cells were incubated for 30 min on ice with secondary Ab linked to Alexa fluor 488 (Molecular Probes, Eugene, Oregon) and then fixed in 1% buffered formalin in PBS. For assessment of total PAR-2 cell staining (surface and intracellular) by flow cytometry, cells were detached from cultures using non-enzymatic techniques, fixed and permeabilized with fixing/permeabilizing solution (Becton Dickinson) at room temperature and then stained with primary and secondary Abs. Analysis was performed on a FACSort flow cytometer (Becton Dickinson) calibrated with Calbrite beads (Becton Dickinson). The distribution of debris and dead cells was assessed on the basis of forward and right angle scatter before proceeding with the analysis. A total of 5–10,000 events were examined using a 488 nm wavelength excitation. Acquired events were analyzed using Cell Quest Software (Becton Dickinson). Results are expressed as % positive cells. Microscopic visualization of total and surface staining was performed after depositing labeled cells onto slides by cytocentrifugation, and counterstaining with propidium iodide (15 mg per mL).

For total *in situ* PAR-2 cell staining and confocal microscopy (at the Microscopy Imaging Center, SUNY, Stony Brook, New York), cells were grown in chamber slides (Lab Tek, Fisher Scientific), fixed in 10% PBS-buffered formalin and permeabilized with permeabilizing solution (Becton Dickinson) at room temperature and then stained with primary and secondary Abs. Single indirect staining for smooth muscle actin (SMA) employed monoclonal Ab (Sigma, 1 μg per $\leqslant 10^6$ cells) and isotype controls (Becton Dickinson/Pharmigen) and secondary Ab linked to Alexa 488 (Molecular Probes). Double IF labeling for PAR-2 and SMA by either flow cytometry or microscopy employed indirect staining methods for PAR-2 and direct staining with anti-SMA linked to Cy3 (mouse monoclonal IgG, Sigma) or isotype control Ab linked to Cy5 (Jackson Immunoresearch Laboratories, Westgrove, Pennsylvania).

Gel electrophoresis and immunoblotting Cell cultures were washed twice with cold PBS and then immediately lysed with cold

lysis buffer (20 mM sodium phosphate, pH 7.2, 120 mM NaCl, 10 mM NaF, 0.1% SDS, 1.0% NP-40) containing protease and phosphatase inhibitors (5 μg per mL leupeptin, 5 μg per mL E-64, 10 μg per mL chymostatin, 5 μg per mL pepstatin A, 2 μg per mL aprotinin, 1 mM AEBSF, 1 mM 1,10-phenanthroline, 1 mM EGTA, 1 mM EDTA, and 1 mM sodium orthovanadate). Fibroblast cultures were lysed at 80%–100% confluence, using approximately 1 mL of lysis buffer per 2×10^6 cells, by scraping the bottom of the flask with a rubber policeman to ensure thorough extraction of the monolayer. Lysates were cleared by centrifugation at 10,000 rpm for 10 min at 4°C. Protein concentration in cell lysates was determined with the BCA protein assay (Pierce, Rockford, Illinois), using the supplied BSA as a standard. Lysate aliquots were prepared for electrophoresis in Laemmeli sample buffer containing 5% 2-mercaptoethanol and 2 M urea. Equal amounts of protein from each lysate sample, typically 3–10 μg, were separated on a 10% or 12% Tris-HCl Ready Gel (Bio-Rad Laboratories, Hercules, California) and then electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, Massachusetts). Membrane blots were washed briefly in PBS containing 0.1% Tween-20 (PBS/Tw), blocked in PBS/Tw plus 1% non-fat dry milk and then incubated at room temperature for 60 min with primary antibody diluted in PBS/Tw plus 0.1% non-fat milk and 30 μg per mL rabbit IgG. Immunoblots were then washed and incubated at room temperature for 40 min with alkaline phosphatase conjugate (rabbit anti-goat; Zymed Laboratories, South San Francisco, California) diluted 1:3000 and stained by incubation with BCIP/NBT substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland). The molecular weights of stained bands on immunoblots were estimated from a standard curve based on the relative mobilities of prestained SDS-PAGE standards (Bio-Rad Laboratories).

Goat polyclonal antibodies, specifically recognizing the amino terminus of human PAR-2, designated antibody PAR-2 (N-19), and the carboxy terminus, designated antibody PAR-2 (C-17), and PAR-2 antigenic blocking peptides were purchased from Santa Cruz Biotechnology. The specificity of the PAR-2 antibodies was determined by pre-absorbing the antibodies with the PAR-2 blocking peptide at 4°C for 2 h prior to performing the immunoblots and comparing the staining patterns to the standard immunoblot conditions.

Induction of PAR-2 expression Two different experimental protocols were used to test the effects of growth factors and cytokines on expression of PAR-2 by normal human dermal fibroblasts. The essential difference between the experiments was the methodology used to produce quiescent cell cultures. In some experments, fibroblasts were seeded into culture flasks at a starting density of about 12,000 cells per cm^2. The cells were grown for 9 d in their original medium (D'Andrea *et al*, 2001), washed three times with SF-MEM, and then exposed to SF-MEM containing the specified factors or cytokines and incubated for 72 h. In other experiments, fibroblasts were seeded into 6-well plates at a similar starting density, but these cells were only grown for 2 d in the original medium, and then they were washed twice with PBS, fed SF-MEM, starved for 2 d, and then exposed to SF-MEM containing growth factors or cytokines and incubated for 72 h. At the end of the treatment period, the effects of the various factors on the expression of PAR-2 were analyzed using the immunoblotting procedure described above. TGF-β1 purified from human platelets, recombinant human PDGF-BB, recombinant human IL-1, and recombinant human TNF-α were purchased from R&D Systems (Minneapolis, Minnesota).

RT-PCR assay Total cellular RNA was extracted from the cells using TRIzol (Invitrogen, Carlsbad, California) according to manufacturer's instructions. Oligo dT-primed reverse transcription was performed in 2 μg of total RNA in a 20 μl reaction volume (Gene Amp RNA PCR, Applied Biosystems, Foster City, California). The primers used for PCR were as follows: PAR-2 forward primer: 5′- TTG ATG GCA CAT CCG ACG TC-3′, PAR-2 reverse primer: 5′- AAT ACC TCT GCA CAC TGA GGC AG-3′; GAPDH forward primer: 5′- CTA CAA TGA GCT GCG TGT GG-3′, GAPDH reverse primer: 5′- AAG GAA GGC TGG AAG AGT GC-3′. The primers were constructed to generate fragment of 385 bp for PAR-2 and 527 bp for GAPDH. Cycling conditions were as follows: 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 35 cycles. Amplified samples were visualized on 2% agarose gel stained with ethidium bromide and photographed under UV light.

Regulation of PAR-2 expression by matrix Cells grown on collagen: Two fibroblast cell lines, 4392A and 2261B, were cultured in DMEM with 10% FBS, 1% PSM at a concentration of 5×10^4 cells per well. Cells were grown on either untreated tissue culture plastic or dishes coated with type I collagen or fibronectin (Biocoat cellware, Becton Dickinson). The cells were allowed to attach overnight and then starved for 24 h in DMEM with 2% BSA and incubated up to 72 h at 37°C, 5% CO_2 in DMEM culture media before washing and lysing with 1% octylglucoside in 50 mM HEPES pH 7.4 to which was added protease and phosphatase inhibitors as previously noted. Protein concentration was determined with the BCA protein assay (Pierce) and lysate was loaded onto 12% SDS gels for PAGE (Bio-Rad, Hercules, California) and then transferred for immunoblotting.

Cells grown in collagen matrix Collagen gels were made by adding collagen (Vitrogen, Cohesion, Palo Alto, California) that had been neutralized with NaOH to fibroblasts (4392A or 2261B) for a final concentration of 5×10^4 cells/well in six-well tissue culture plates (Falcon, Becton Dickinson) with or without PDGF-BB 30 ng per mL (R&D Systems) and then the mixture was allowed to gel. The gels were suspended in DMEM, 10% FCS, 1% PSM and incubated 24 and 48 h at 37°C, 5% CO_2. The collagen gels were dispersed with collagenase 10 ug per mL (Sigma, St Louis, MO) to liberate the cells which were then washed and lysed with 1% octylglucoside in 50 mM HEPES pH 7.4 to which was added protease and phosphatase inhibitors as previously noted. Protein concentration was determined with the BCA protein assay (Pierce) and lysate was loaded onto 12% SDS gels for PAGE (Bio-Rad, Hercules, CA) and then transferred to membranes for immunoblotting.

Cells grown in an organotypic skin equivalent In order to mimic an *in vivo* environment for PAR-2 expression, organotypic skin cultures were prepared as described previously (Garlick and Taichman, 1994; Gruber *et al*, 1997). The culture system used consisted of a polymerized collagen gel matrix with embedded human foreskin fibroblasts that served as a biologic substructure to support growth and stratification of human foreskin keratinocytes. Organotypic cultures or rafts were grown submerged for 5 d and then raised to the air interface for another 5 d to allow for complete stratification and differentiation of surface keratinocytes. We have used this skin-equivalent culture system previously to study interactions between mast cells and dermal fibroblasts since it manifests epithelial stratification and mimics other properties of living skin (Gruber *et al*, 1997).

Statistical analyses Statistical differences between flow cytometry samples were analyzed by using a two-tailed Student's *t* test. The results of the immunoblots were analyzed by densitometry from scanned images and the absorbance units per band were quantified using NIH Image software.

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